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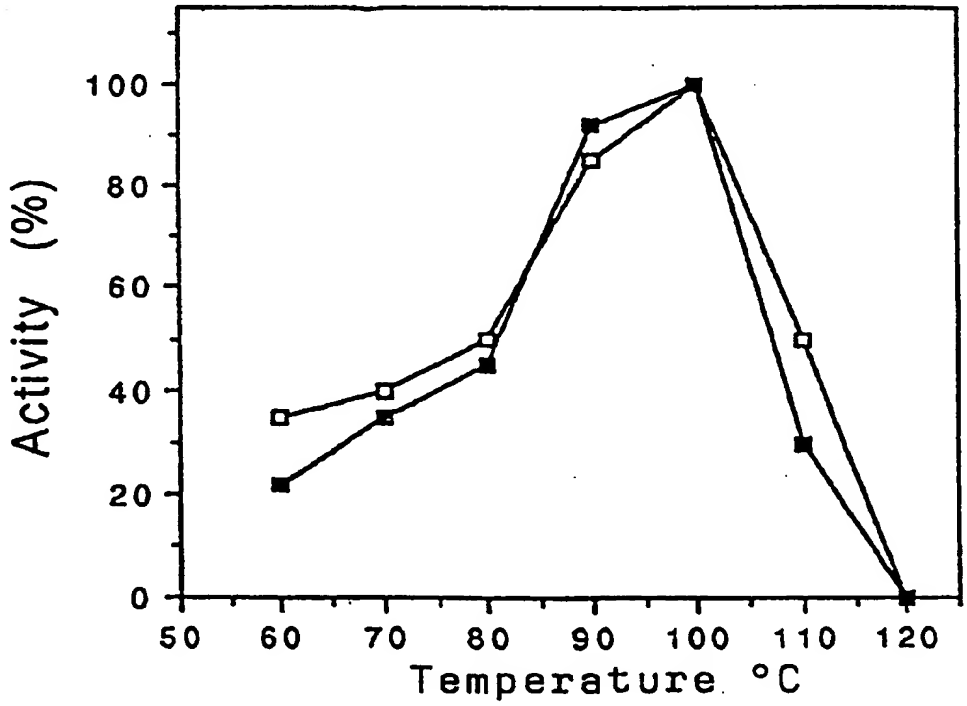
(54) Title: DESULFUROCOCCUS AMYLASE AND PULLULANASE



(57) Abstract

The present invention relates to *Desulfurococcus* amylase and pullulanase preparations and their use in producing sweeteners and ethanol from starch.

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DESULFUROCOCCUS AMYLASE AND PULLULANASE

FIELD OF INVENTION

The present invention relates to a novel thermostable amylase and a novel thermostable pullulanase and their use in the production of sweeteners and ethanol from starch.

BACKGROUND OF THE INVENTION

The production of sweeteners from starch has been largely improved by application of different microbial enzymes to obtain better quality and yields, but the necessity of performing several steps of the starch-hydrolysing process at elevated temperatures means that there is still a need for new starch-hydrolysing enzymes with increased thermal stability.

It is known that Pyrococcus, e.g. Pyrococcus wosei and Pyrococcus furiosus, for reference see Arch. Microbiol. 155, 1991, pp. 572-578, and Appl. Env. Microbiol. 56, 1990, pp.1985-1991, can produce highly thermostable amylases.

It is the object of this invention to provide an amylase and a pullulanase with temperature optimum at 80°C or above 80°C.

SUMMARY OF THE INVENTION

We have unexpectedly found that a novel thermostable amylase and a novel thermostable pullulanase can be obtained from Desulfurococcus mucosus, a strain not previously reported to produce thermostable amylase and pullulanase; these new enzymes have temperature optimum around 100°C.

Accordingly, the invention provides an amylase preparation, characterized by being producible by cultivation of an amylase producing strain of Desulfurococcus mucosus, and a pullulanase preparation, characterized by being producible by cultivation of a pullulanase producing strain of Desulfurococcus mucosus.

BRIEF DESCRIPTION OF DRAWINGS

The present invention is further illustrated by reference to the accompanying drawings, in which:

Fig. 1 shows the relative activity (% rel.) of an amylase (□) and a pullulanase (■) of the invention at various temperatures (determined at pH 5.5 with starch and pullulan, respectively, as substrate).

Fig. 2 shows the relative activity (% rel.) of an amylase (□) and a pullulanase (■) of the invention at various pH, determined at 90°C with starch and pullulan, respectively, as substrate.

DETAILED DISCLOSURE OF THE INVENTION

The Microorganism

According to the invention, amylase is derived from an amylase producing strain of Desulfurococcus mucosus and pullulanase is derived from a pullulanase producing strain of Desulfurococcus mucosus.

A strain representative of Desulfurococcus mucosus has been made publicly available under Accession No. DSM 2162. The number is published in the DSM Catalogue of Strains, 1993.

Production of Amylase and Pullulanase

Amylase and pullulanase of the invention may be produced by anaerobic cultivation of the above mentioned strain on a nutrient medium containing suitable carbon and nitrogen sources, such media being known in the art. Anaerobic conditions may be achieved during the preparation of media by sparging with N_2 and following the anaerobic techniques as described by Balch and Wolfe in Appl. Env. Microbiol. 32, 1976, pp. 781-791.

Alternatively, amylase and pullulanase of the invention can be produced by aerobic cultivation of a transformed host organism containing the appropriate genetic information from the above mentioned strain. Such transformants

can be prepared and cultivated by methods known in the art.

The amylase and the pullulanase may be recovered by removing the cells from the fermentation medium (e.g. by centrifugation or filtration) and then concentrating the broth (e.g. by ultrafiltration). If desired, the amylase and the pullulanase may be further purified by known methods.

Immunochemical Properties

The enzymes of the invention have immunochemical properties identical or partially identical (i.e. at least partially identical) to those of an enzyme derived from the strain Desulfurococcus mucosus, DSM 2162.

The immunochemical properties can be determined immunologically by cross-reaction identity tests. The identity tests can be performed by the well-known Ouchterlony double immunodiffusion procedure or by tandem crossed immunoelectrophoresis according to Axelsen N.H.; Handbook of Immunoprecipitation-in-Gel Techniques; Blackwell Scientific Publications (1983), chapters 5 and 14. The terms "antigenic identity" and "partial antigenic identity" are described in the same book, Chapters 5, 19 and 20.

Monospecific antisera are generated according to the above mentioned method by immunizing rabbits with the purified enzymes of the invention. The immunogens are mixed with Freund's adjuvant and injected subcutaneously into rabbits every second week. Antisera are obtained after a total immunization period of 8 weeks, and immunoglobulins are prepared therefrom as described by Axelsen N.H., supra.

The Enzymes

An amylase of the invention can be characterized by having amylase activity at temperatures of from below 60°C to approximately 120°C, having activity optimum at temperatures in the range 95-105°C, determined at pH 5.5 with starch as substrate. The amylase can also be characterized by having amylase activity at pH values of from below pH 4.0 to approximately pH 11.0, having optimum in the range pH 5.5 to pH

6.5, determined at 90°C with starch as substrate.

A pullulanase of the invention can be characterized by having pullulanase activity at temperatures of from below 60°C to approximately 120°C, having activity optimum at temperatures in the range 90-105°C, determined at pH 5.5 with pullulan as substrate. The pullulanase can also be characterized by having pullulanase activity at pH values of from below pH 4.0 to approximately pH 9.0, having optimum in the range pH 4.8 to pH 5.8, determined at 90°C with pullulan as substrate.

Determination of Amylase Activity

Amylase activity is determined by measuring the amount of reducing sugar released during the incubation with starch. One unit (U) of amylase activity is defined as the amount of amylase that releases 1 μmole of reducing sugar (as maltose standard) per min. under the following assay conditions: A 0.05 ml volume of 1% soluble starch is added to 0.05 ml of 0.1 M sodium acetate buffer pH 5.5. 25 μl of enzyme solution are added to this mixture and the sample is incubated at 90°C for 30 min. The reaction is stopped by cooling on ice, and the amount of reducing sugar is determined by dinitrosalicylic acid. Sample blanks are used to correct for non-enzymatic release of reducing sugar.

Determination of Pullulanase Activity

Pullulanase activity is determined by measuring the amount of reducing sugar released during the incubation with pullulan. One unit (U) of pullulanase activity is defined as the amount of pullulanase that releases 1 μmole of reducing sugar (as maltose standard) per min. under the following assay conditions: A 0.05 ml volume of 1% pullulan is added to 0.05 ml of 0.1 M sodium acetate buffer pH 5.5. 25 μl of enzyme solution are added to this mixture and the sample is incubated at 90°C for 30 min. The reaction is stopped by cooling on ice, and the amount of reducing sugar is determined by dinitrosalicylic acid. Sample blanks are used to correct for nonenzymatic

release of reducing sugar.

Industrial Applications

The enzymes of this invention possess valuable properties allowing for various industrial applications. In particular the enzymes, in being thermostable, find potential application in the production of sweeteners and ethanol from starch. Conditions for conventional starch converting processes and liquefaction and/or saccharification processes are described in for instance US Patent No. 3,912,590 and EP patent publications Nos. 252,730 and 63,909.

The following example further illustrates the present invention, and it is not intended to be in any way limiting to the scope of the invention as claimed.

EXAMPLE 1

Cultivation

The strain Desulfurococcus mucosus, DSM 2162, was recultured from glycerol-preserved cells using the medium recommended by the Deutsche Sammlung von Mikroorganismen (DSM). The microorganisms were grown in 1 liter batch cultures under the following conditions: Medium: DSM184 (DSM184 is described in DSM Catalogue of Strains, 1993), pH 5.8, temp. 85°C; in the medium sulphur and tryptone were omitted and starch (0.5% w/v) was added as the only carbohydrate; yeast extract concentration was 0.1% (w/v). The cell density achieved in this medium was $\geq 10^8$ cells/ml. Anaerobic conditions were achieved during the preparation of media by sparging with N_2 and following the techniques as described by Balch in Appl. Env. Microbiol. 32, 1976, pp. 781-791.

After cultivation the culture fluid was centrifuged at 12.000 x g for 30 min. at 4°C, and the cell free supernatant was concentrated up to 100-fold using an Amicon Ultrafiltration System. The cell pellet was resuspended in 50 mM sodium acetate buffer pH 5.5 and sonicated three times for 3 min. at 50% duty

cycle by a BRANSON 450 sonifier. The cell debris was separated from the supernatant after centrifugation at 10.000 x g for 30 min. at 4°C.

The following total activity (U) in both supernatant and cell extract was found:

Amylase activity: 2.0 U/l
Pullulanase activity: 0.8 U/l

Temperature Optima

Temperature optima were determined by incubation of samples for 30 minutes at pH 5.5 at temperatures from 60°C to 120°C. The incubation was conducted in closed Hungate tubes in order to prevent boiling of the solution.

Fig. 1 shows the result (Amylase (▫) and pullulanase (▪)).

pH Optima

To determine pH optima, Universal buffer (Britten and Robinson) was used to obtain values from pH 4.0 to pH 11.0. Samples were incubated for 30 minutes at 90°C at the pH in question.

Fig. 2 shows the result (Amylase (▫) and pullulanase (▪)).

CLAIMS

1. An amylase preparation, characterized by being producible by cultivation of an amylase producing strain of Desulfurococcus mucosus.

2. An amylase preparation according to claim 1, wherein said amylase producing strain is Desulfurococcus mucosus, DSM 2162.

3. An amylase according to claim 2, further characterized by:

(a) Activity optimum in the range pH 5.5 to pH 6.5, determined at 90°C with starch as substrate;

(b) Activity optimum at temperatures in the range 95-105°C, determined at pH 5.5 with starch as substrate.

4. A pullulanase preparation, characterized by being producible by cultivation of a pullulanase producing strain of Desulfurococcus mucosus.

5. A pullulanase preparation according to claim 4, wherein said pullulanase producing strain is Desulfurococcus mucosus, DSM 2162.

6. A pullulanase preparation according to claim 5, further characterized by:

(a) Activity optimum in the range pH 4.8 to pH 5.8, determined at 90°C with pullulan as substrate;

(b) Activity optimum at temperatures in the range 90-105°C, determined at pH 5.5 with pullulan as substrate.

7. The use of the amylase according to any of claims 1-3 in a process of producing sweeteners from starch.

8. The use of the amylase according to any of claims 1-3 in a process of producing ethanol from starch.

9. The use of the pullulanase according to any of claims 4-6 in a process of producing sweeteners from starch.

10. The use of the pullulanase according to any of claims 4-6 in a process of producing ethanol from starch.

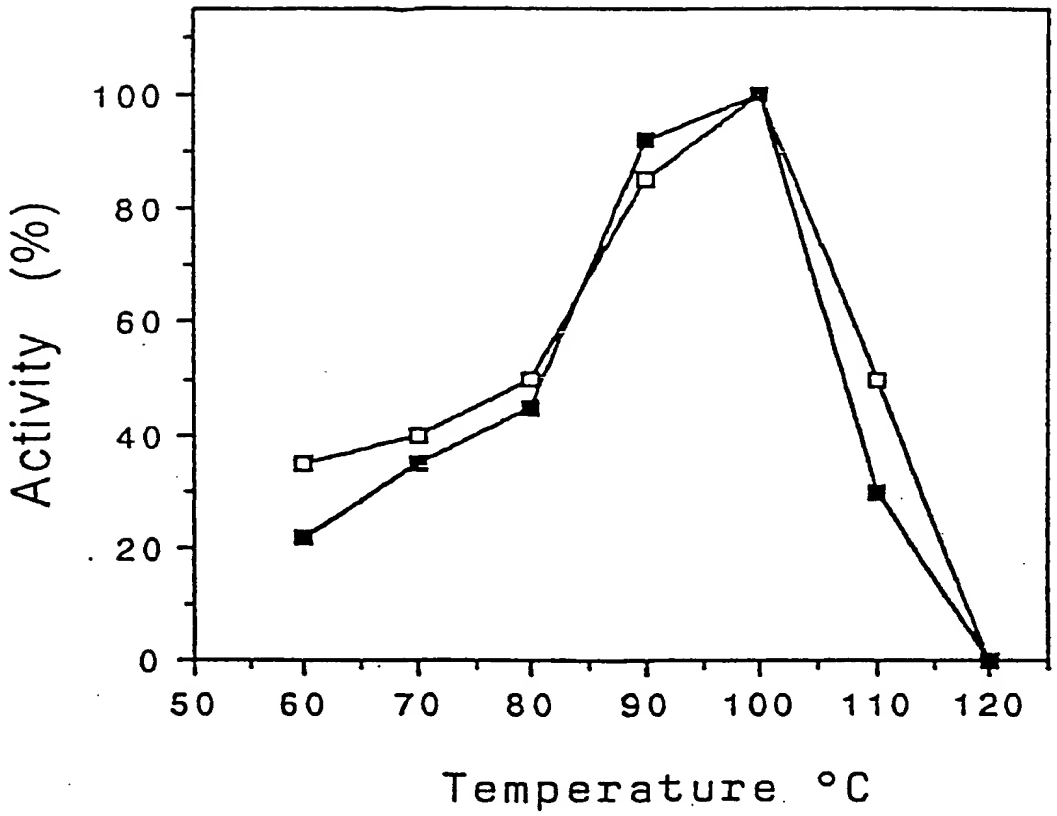
Activity (%)
100
80
60
40
20
0
50
60
70
80
90
100
110
120
Temperature °C

Fig 1

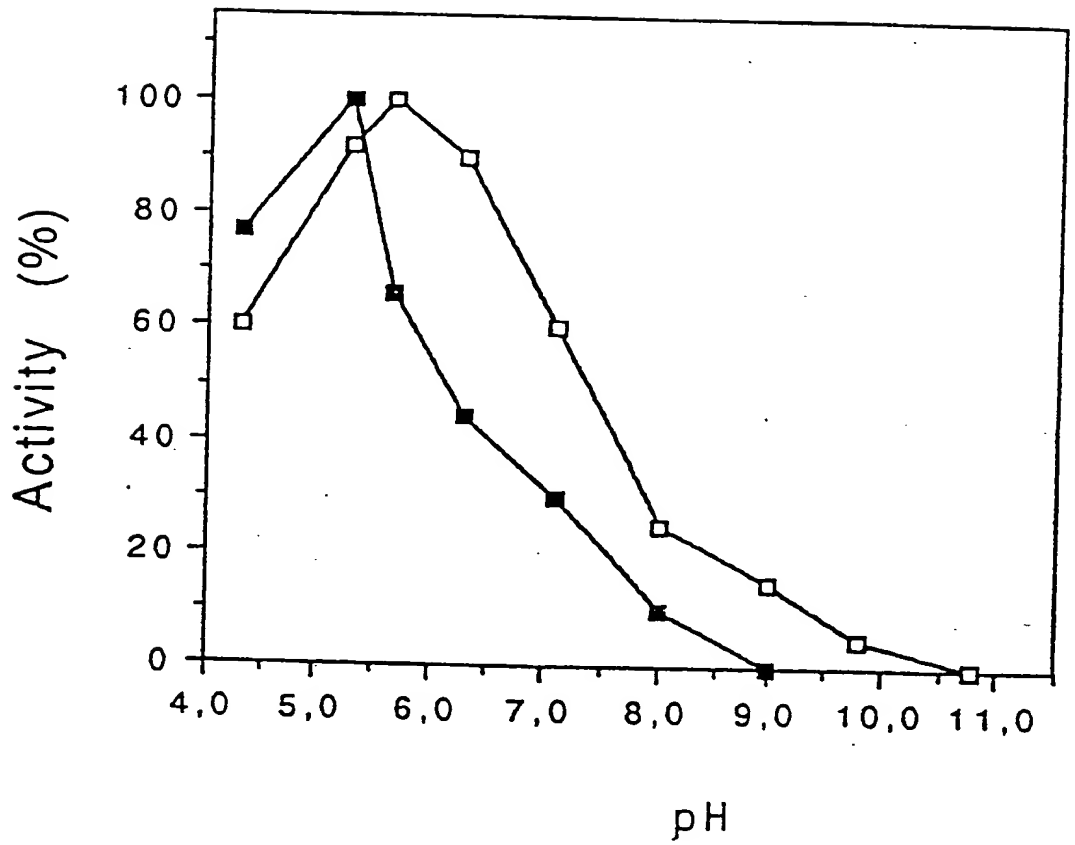

Fig. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 95/00098

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/26, C12N 9/44
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS, WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TIBTECH, Volume 10, Sept 1992, Don A. Cowan, "Biotechnology of the Archaea" page 315 --	1,4
A	National Library of Medicine (NLM), Medline, Medline accession no. 94079331, Adams MW: "Enzymes and proteins from organisms that grow near and above 100 degrees C", & Annu Rev Microbiol 1993;47:627-58 --	1,4

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
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"E" erlier document but published on or after the international filing date
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15 June 1995	22 -06- 1995
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International application No.
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Dialog Information Services, file 357, Derwent Biotechnology Abs, Dialog accession no. 170578, DBA accession no. 94-13129, Rossi M et al: "Extremophiles in biotechnology - production and application of e.g. thermophilic bacterium and its enzyme (conference paper)", Prog. Biotechnol. (9, Pt.1,255-62) 1994 --	1-10
P,X	Dialog Information Services, file 55, BIOSIS PREVIEWS, Dialog accession no. 11491261, BIOSIS no. 98091261, Canganella F et al: "Characterization of amylolytic and pullulytic enzymes from thermophilic archaea and from a new Fervidobacterium species", & Applied Microbiology and Biotechnology 42 (2-3). 1994. 239-245 -- --------	1-10